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Case study

Pancreatic ductal adenocarcinoma in hereditary diffuse gastric cancer. A case report

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Summary Hereditary diffuse gastric cancer is an autosomal dominant cancer syndrome characterized by highly penetrant diffuse gastric cancer. It is caused by germ line mutations in *CDH1*, encoding the cell-cell adhesion protein E-cadherin. Pancreatic ductal adenocarcinoma is one of the most dismal malignancies in humans. Although absent E-cadherin expression in pancreatic ductal adenocarcinoma is related to a higher tumor grade and a worse prognosis, there have been no reports of pancreatic ductal adenocarcinoma associated with hereditary diffuse gastric cancer. Here, we describe a patient with hereditary diffuse gastric cancer who was subsequently diagnosed with pancreatic ductal adenocarcinoma. To investigate if the previously identified *CDH1* germ line mutation initiated pancreatic ductal adenocarcinoma development, we performed mutational and proteomic analyses. We conclude that the pancreatic ductal adenocarcinoma did not occur in the context of the germ line *CDH1* mutation but rather appeared as a sporadic event. Immunohistochemistry ultimately proved to be the most valuable tool of investigation as persistent *CDH1* staining in the pancreatic ductal adenocarcinoma unequivocally revealed E-cadherin expression.

1. Introduction

Hereditary diffuse gastric cancer (HDGC) is an autosomal dominant cancer syndrome [1] characterized by diffuse gastric cancer (DGC) that presents with high penetrance. The risk of developing DGC is estimated to be 67% in men and 83% in women by the age of 80 years [2]. The *CDH1* gene, encoding E-cadherin, is the only known gene associated with HDGC, and in 25% to 50% of patients with HDGC, a germ line mutation in *CDH1* has been identified. To date, approximately 100 different germ line mutations in *CDH1* have been described in more than 150 HDGC families [3,4]. Because of the high penetrance, aggressive behavior, and difficulty of detecting tumor on surveillance endoscopy, a

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prophylactic total gastrectomy is recommended for *CDH1* germ line mutation carriers [5]. This operation is carried out in the third decade of life.

Pancreatic ductal adenocarcinoma (PDAC) is known for its poor prognosis, with a 5-year survival of less than 5% and a median survival of only 6 months [6]. Because most patients present with locally advanced or distant metastatic disease, palliative treatment is often the only viable option. Only 10% to 20% of patients with PDAC qualify for surgical resection, which remains the only curative treatment. Of note, many sporadic mutations have been described in PDAC, including mutations in the *KRAS2* oncogene and the tumor suppressor genes *CDKN2A*/*p16*, *TP53*, and *SMAD4* [7]. With respect to E-cadherin in PDAC, loss of expression is related to a higher tumor grade and a worse prognosis [8]. However, there have been no reports of a PDAC in association with HDGC. Moreover, there is as yet no evidence for an increased risk of PDAC in patients with a germ line *CDH1* mutation.

In this case report, we describe a patient recently diagnosed with PDAC who harbored a previously identified *CDH1* germ line mutation and had a history of multifocal intramucosal diffuse gastric carcinoma that was diagnosed in the prophylactic gastrectomy specimen. To investigate whether the pancreatic tumor was related to the germ line *CDH1* mutation, we performed immunohistochemistry (IHC) for E-cadherin on the gastric tumor, the pancreatic tumor, and normal control tissue. Moreover, extensive mutation and methylation analysis was performed in search of the "second genetic hit," which may have eliminated *CDH1* signaling. Furthermore, to further characterize the PDAC, *KRAS2*, and *TP53* mutation, analysis was performed on the pancreatic tumor samples.

2. Case history

A 56-year-old man presented to the emergency department with intense epigastric pain, diarrhea, and dark urine. Physical examination revealed jaundice and a palpable liver 2 fingers under the rib cage. His clinical history included a germ line mutation in *CDH1* for which a prophylactic total gastrectomy had been carried out 2 years prior. Pathological examination of the resected stomach had revealed a multifocal diffuse growing poorly differentiated mucus-producing adenocarcinoma, with foci varying in size from 1 to 8 mm that were limited to the lamina propria (intramucosal carcinoma). Four family members had died of gastric cancer between the ages of 43 and 56 years. There was no family history of pancreatic cancer.

A computed tomography scan was performed that revealed enlarged bile and pancreatic ducts and a mass in the head of pancreas, suspicious for a malignant pancreatic tumor. A Whipple's pancreatoduodenectomy was performed. Examination of the resected head of pancreas revealed a 3-cm mass that, on microscopic examination, was diagnosed as a moderately to poorly differentiated PDAC. The malignant process extended into the duodenal wall and was present less than 1 mm from the duodenal resection margin. All other resection margins were free of tumor. Of the 16 excised lymph nodes, 3 contained metastases, warranting the staging T3N1Mx or IIB. Adjuvant therapy was initiated consisting of several runs of gemcitabine. However, 3 months later, a computed tomography scan revealed a recurrent lesion in the pancreas and multiple lesions in the liver, suspicious for metastatic tumors, and treatment with curative intent was ceased. The patient was treated for his pain and discharged home where he died 2 months later.

3. Materials and methods

Paraffin blocks of both the gastric tumor and the PDAC were obtained. Neoplastic cells from the PDAC were isolated using manual microdissection. With use of laser microdissection (Carl Zeiss, Jena, Germany), the malignant cells of the gastrectomy specimen were isolated from the lamina propria. Normal gastric epithelium and a non-malignant lymph node were included as control samples. Genomic DNA was isolated using the Qiamp DNA minikit (Qiagen, Venlo, Netherlands) according to manufacturer's protocol or, in the case of the gastric cells, by direct lyses with Proteinase K (Roche, Basel, Switzerland).

3.1. IHC

IHC was performed on formalin-fixed, paraffin-embedded tissue samples as previously described [9]. Two different E-cadherin antibodies were used: E-cadherin antibody, dilution 1:100, (ab1416; Abcam, Cambridge, UK), which is directed at the extracellular part of the protein (exon-8), and E-cadherin, clone 36, dilution 1:200, (BD Bioscience, Breda, The Netherlands), which binds the cytoplasmic domain.

3.2. E-cadherin mutation analysis

To confirm the germ line mutation earlier identified and possibly identify a somatic mutation in the wild type (WT)-allele, the complete *CDH1* gene was sequenced in all samples. Sequencing was performed as previously described [9]. Forward and reverse primers for each exon were designed (sequences available upon request). The sequencing reaction mix was run on the 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were analyzed using the CODONCODE ALIGNER software (Codoncode Corperation, Dedham, MA).

3.3. Loss of heterozygosity

To evaluate loss of heterozygosity (LOH) of E-cadherin, 1 microsatellite marker was used, located on chromosome 16q22 (D16s2624) around the *CDH1* locus. Polymerase chain reaction (PCR) was performed (primer sequences and cycling conditions available upon request). Analysis was carried out using an automated ABI 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan 500 ROX Size Standard and the manufacturer's Genescan 3.1.2 software (Applied Biosystems). LOH was assumed if the allelic imbalance factor was greater than 1.6 or smaller than 0.6.

3.4. Methylation analysis

A methylation-specific PCR (ms-PCR) reaction was performed to evaluate promotor methylation of the *CDH1* gene using the Epitect Bisulfite kit (Qiagen) according to the protocol provided by the manufacturer. After bisulfite treatment, PCR reactions were performed. Primers specific for either methylated or unmethylated templates were designed (sequences and cycling conditions available upon request). Sequencing was performed as described above.

Because ms-PCR did not produce reliable results, another methylation assay was performed. For this method, the SALSA MLPA KIT ME004-A1 Tumour Suppressor kit (MRC-Holland, Amsterdam, The Netherlands) was used. This kit contains 31 probes that recognize CpG islands in 27 different tumor suppressor genes including *CDH1*. Analysis was performed as described by the manufacturer. Genescan analysis was performed as described above, and samples were analyzed using the Cofalyzer software (MRC Holland).

3.5. TP53/KRAS2 mutation analysis

To evaluate mutation status of *TP53* and *KRAS2* in the PDAC sample, sequencing was performed. Primers for exon 4-9 of *TP53* and exon 1-2 of *KRAS2* were designed (sequences and PCR cycling conditions available upon request). PCR products were purified using a combination of exonuclease I (Westburg, Leusden, The Netherlands) and shrimp alkaline phosphatase (GE Healthcare, Chalfont St. Giles, UK). Sequencing was performed as described above and analyzed using Mutation Surveyor (Softgenetics, State College, PA).

4. Results

4.1. IHC

Immunohistochemical staining showed the same labeling pattern for E-cadherin in all gastric tumors. Membranous staining was clearly diminished compared with the normal epithelium surrounding the malignant cells, suggesting a defect in E-cadherin signaling in the gastric lesions (Fig. 1C and D). Cytoplasmic staining was granular and focally positive. Exon-8 skipping as a second genetic hit, often observed in sporadic DGC [10], was shown to be absent, as one of the E-cadherin antibodies used specifically recognized exon-8. In the PDAC sample, E-cadherin staining was strong with both antibodies, and there was no decrease in intensity compared with normal epithelial cells suggesting intact E-cadherin signaling (Fig. 1E and F).

4.2. Mutation analysis

Mutation analysis for *CDH1* did not identify any new mutations. The germ line mutation detected previously was confirmed in all samples. The mutation was located at the splice site of exon 10 and intron 10 and consisted of an insertion of a T-nucleotide just 2 bases after exon 10: 1565+2insT, as shown in Fig. 1A.

4.3. LOH

To further specify E-cadherin signaling, LOH analysis was performed. The allelic imbalance factor was calculated at 1.21 for the gastric tumor samples and 1.00 for the PDAC sample, confirming the absence of LOH in all samples. In addition, LOH was ruled out by the mutation analysis of *CDH1*, which revealed a heterozygous SNP in exon 13 (RS1801552) in both the gastric and the pancreatic samples.

4.4. Methylation analysis

An ms-PCR did not produce reliable results. Therefore, another technique, methylation-specific multiplex ligand-dependent probe-amplification, was tried. Unfortunately, although this reaction was repeated multiple times, it did not result in interpretable data either, presumably as the amount of DNA obtained from the gastric cells was not sufficient to perform further testing. No E-cadherin promoter methylation was found in the PDAC.

4.5. Analysis of the PDAC for KRAS2 and TP53

KRAS2 mutation analysis of the PDAC sample revealed a point mutation in exon 1, codon 12, and G12D. In *TP53*, a mutation was found as well. Sequencing of exon 7 revealed the TAT-TGT missense mutation at codon 238, responsible for Cys to Tyr substitution (C238T).

5. Discussion

Decreased E-cadherin expression has been described in numerous different types of malignancies, including pancreatic

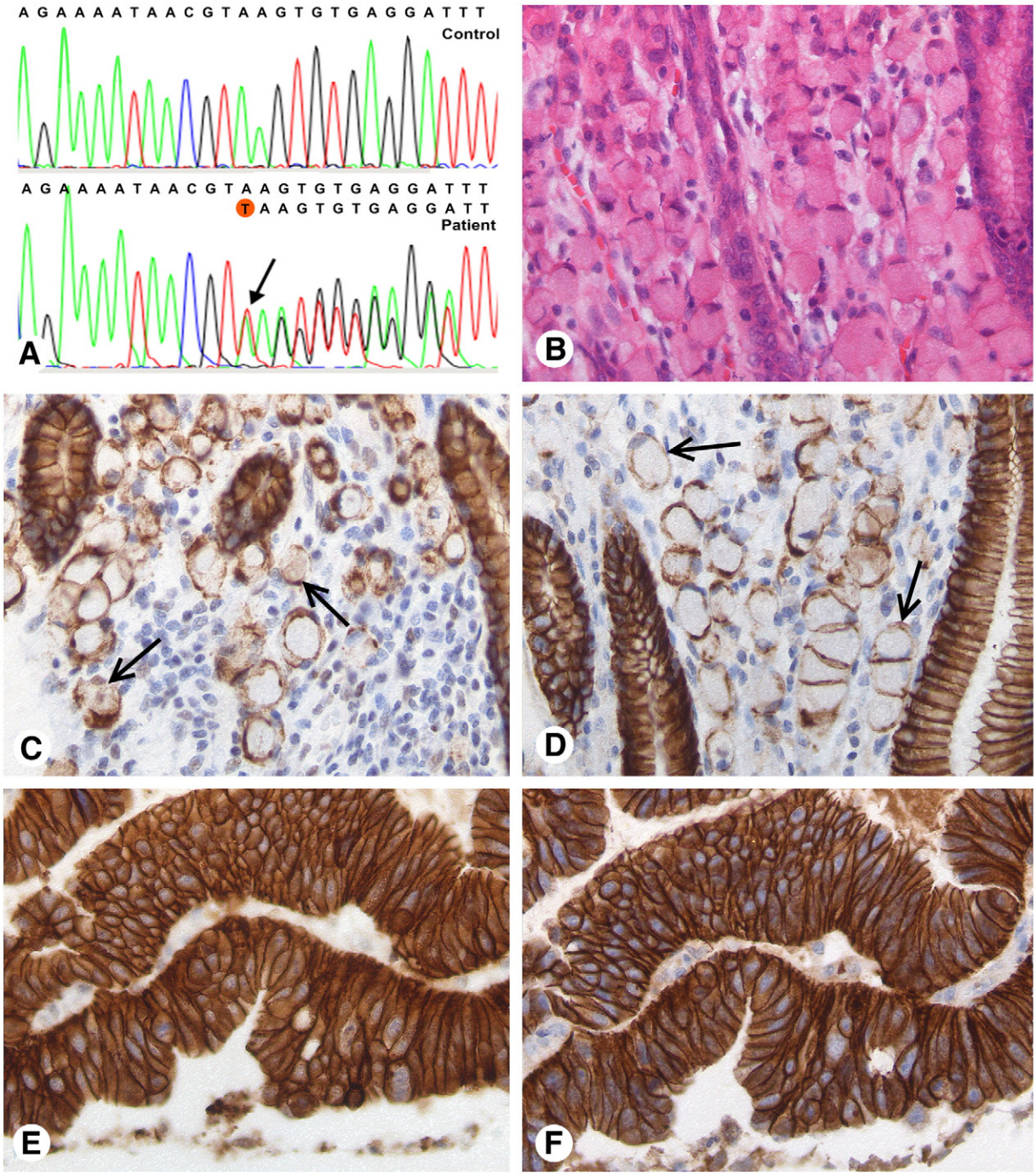

Fig. 1 A, Mutation analysis *CDH-1* showing a mutation in exon 10, 1565 +2insT in the patient. Arrow indicates mutation. B, Hematoxylin-eosin stained gastric resection specimen clearly depicting the DGC cells. C and D, E-cadherin IHC on DGC cells using 2 different antibodies both showing a clear decrease in membranous staining intensity in the signet ring cells compared with the normal gastric epithelium; granular cytoplasmic staining is present in the tumor cells. E and F, E-cadherin IHC on the PDAC using 2 different antibodies showing strong membranous as well as cytoplasmic staining suggesting unaltered E-cadherin expression in the malignant cells.

cancer. However, no data suggest that patients with HDGC have a higher chance of developing pancreatic tumors, implying that sole loss of E-cadherin expression is not sufficient to initiate tumorigenesis in the pancreas. Nevertheless, there is a strong suggestion that diminished E-cadherin expression increases invasive potential and worsens prognosis in PDAC [8]. Therefore, it seemed plausible that the germ line *CDH1* mutation in this patient may have played a role in the development of his pancreatic tumor.

IHC for E-cadherin in the gastric samples showed a clearly diminished labeling intensity in the malignant cells, suggesting a defect in E-cadherin signaling. Although we

tried to identify the mechanism for the somatic knock out of the WT-allele in the gastric tumor cells, this was not possible. Sequencing showed only the previously known germ line mutation. LOH was also not the cause for the decrease in E-cadherin expression. Methylation of the promotor, the most common second genetic hit in HDGC [11], could not be ruled out because methylation analysis was incomplete because of technical difficulties. However, the persistent yet decreased E-cadherin staining in the gastric tumors with IHC suggests that posttranslational modifications are more likely to have caused a somatic knock out.

The pattern of E-cadherin staining in the gastric samples observed with IHC has been described previously. In 2009, Humar et al [12] found residual activity of E-cadherin in patients with HDGC, even with a second genetic hit consisting of promoter methylation. Barber et al [13] described their search for the mechanism of somatic inactivation of the wild-type allele and found persistent yet reduced staining in almost all cases. Remarkably, one of their cases exhibited a mutation at almost the exact same location as the patient described above, 1565 + 1G > T, and similar to the patient described in this case report, E-cadherin labeling was decreased, yet no LOH or promotor methylation was identified.

The persistent E-cadherin labeling with IHC with no evidence of a decrease in intensity clearly showed the presence of the wild-type allele in the PDAC sample, thereby excluding *CDH1* as a PDAC tumor inducer. This conclusion can be drawn solely upon the results from the IHC but is strongly supported by the molecular analyses carried out on *KRAS2* and *TP53*, as the alterations found in these genes are characteristic for sporadic PDACs.

As shown by other groups, identifying the second genetic hit remains difficult in HDGC, and this study emphasizes once more the value of IHC. Although more elaborate and refined techniques have been developed, we highlight the vital importance and relevance of IHC that, with the use of a reliable antibody, enabled a conclusion to be drawn based on protein expression. Although the exact mechanism for E-cadherin loss was not identified, the knowledge that this did not influence the development of the PDAC is crucial for the patient's family, physicians, and basic scientists.

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